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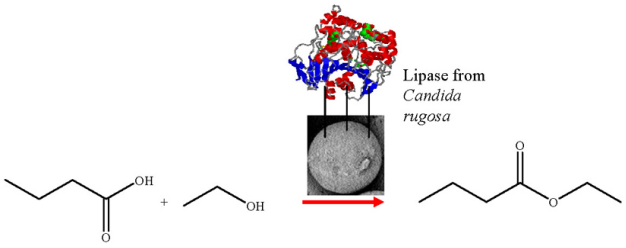
Biosynthesis of ethyl butyrate with immobilized *Candida rugosa* lipase onto modified Eupergit®C

Abstract: Lipase from *Candida rugosa* was immobilized onto the modified Eupergit®C. The support was treated with ethylenediamine and subsequently activated with glutaraldehyde. Enzyme immobilization efficiency was 85%. The optimum pH was close to 6.5 for both the free and immobilized lipase. Immobilized lipase retained its maximum activity in a temperature range of 55 – 60°C. Subsequently, ethyl butyrate synthesis was investigated using immobilized enzyme by esterification of butyric acid with ethanol in solvent-free conditions (23% product yield) and using hexane as a solvent (65% product yield). The acid-alcohol molar ratio and different enzyme amounts were tested as efficient reaction parameters. The biocatalyst maintained 60% of its activity when reused in 8 successive batch reactions in organic solvent. Therefore, the immobilized lipase has demonstrated its potential in practical applications such as short-chain ester synthesis for the food industry.



Keywords: *Candida rugosa* lipase, immobilization, Eupergit®C, esterification, ethyl butyrate

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1 Introduction

Esters of short chain acids and alcohols are known as flavor and fragrance compounds in foods [1-3]. For example, esters are responsible for the aroma of many fruits, e.g., pineapple and banana, and find many applications in the food industry [4-7]. Growing consumer demands for health protection are increasing the requests for natural food ingredients. However, the common sources of natural flavor compounds are not enough to meet these market requests and often their direct extraction from plants involves expensive and low-yielding processes [8]. Therefore, new enzymatic synthesis, and their optimization to obtain these flavor molecules, represents an important alternative for the food industry [9,10]. Lipases (acylglycerol acylhydrolases, EC 3.1.1.3) are enzymes with the ability to catalyze the hydrolysis of oils and fats. Under appropriate reaction conditions, lipases can also catalyze esterification, transesterification, and alcoholysis [11,12]. Lipases display catalytic activity towards a large variety of short chain alcohols and short chain carboxylic acids in ester synthesis reactions [6,13].

Short-chain flavor esters have been generally produced by free and immobilized lipases from various sources in organic solvents [14,15]. Lipase immobilization is known to allow better operation control, easier product recovery, flexibility of reactor design and, in some cases, enhanced operational stability [16,17]. Furthermore, lipase immobilization onto carriers via a linker seems to be a good strategy to avoid steric hindrance and to enhance enzymatic activity [18,19]. As reported in the literature, the development of porous materials have allowed their application in many research areas of epoxy-activated supports as systems to develop simple protocols for enzyme immobilization [20,21].

Although higher conversion yields are achieved in organic solvent, their toxicity is generally a problem for many industrial applications. Moreover, some commonly employed organic solvents are too expensive to allow an industrial scale-up [22]. Several alternatives to facilitate downstream processing have been investigated for ester

synthesis with a minimum number of components in the reaction mixture. Furthermore, the reduced use of organic solvents from the production step can potentially significantly lower the costs and environmental impact. In the literature, there are few studies concerning the production of flavor and fragrance short-chain esters in solvent-free systems by enzymatic synthesis [23,24].

In this study, the immobilization process of *Candida rugosa* lipase onto modified Eupergit®C was optimized and applied to the synthesis of ethyl butyrate in a solvent-free system; this synthesis was compared with the preparation in organic solvent.

2 Methods

2.1 Chemicals

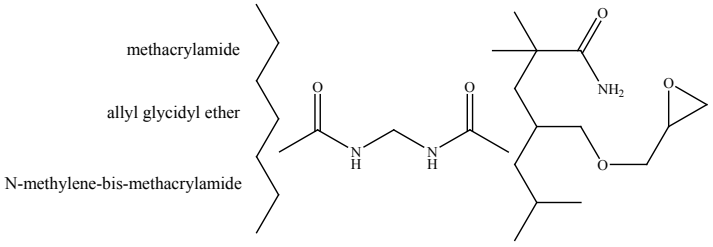
Eupergit®C 250 L lipase (particle size 150 μm, oxirane content ≥800 μmol/g dry support) (Scheme 1), from *Candida rugosa* (Type VII, 1020 U/mg solid), ethylenediamine, ninhydrin reagent (2% solution), glutaraldehyde solution (aqueous solution, 25% (w/w)), butyric acid (≥ 99%), ethanol (≥ 99%), p-nitrophenyl butyrate (≥ 98%), sodium hydroxide, sodium mono-/di-hydrogen phosphate, and anhydrous acetonitrile (99.8%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phenolphthalein (1%) was purchased from Titolchimica (Rovigo, Italy). All chemicals used in this work were analytical grade and were not further purified.

2.2 Functionalization of Eupergit®C

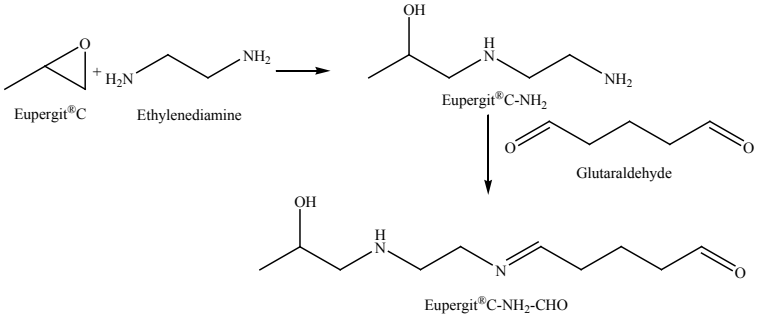
The functionalization procedure consisted of two main steps (Scheme 2): (1) polymer pre-treatment with 1,2-diaminoethane (Eupergit®C-NH_2) and (2) glutaraldehyde activation (Eupergit®C-NH_2-CHO). A specific amount of Eupergit®C (1 g) was added to 10 mL of ethylenediamine (1 M in water, pH = 10) and stirred for 12 h at room temperature. Then, the support was washed with distilled water and dried at 60 °C for 2 h. To activate Eupergit®C-NH_2, 2.5% (v/v) glutaraldehyde in sodium phosphate buffer (25 mL, 50 mM, pH = 7.0) was added, and the reaction was allowed to proceed for 2 h. The support was washed with distilled water on a Büchner funnel and dried at 60 °C for 2 h.

2.2.1 Ninhydrin test

The amount of non-reacted–NH_2 groups after glutaraldehyde activation was determined as described in the literature [25]. Water (200 μL) and ninhydrin reagent (400 μL) were added to 20 mg of Eupergit®C-NH_2 and Eupergit®C-NH_2-CHO. The resulting mixture was heated in a boiling water bath for 30 min, and then cooled at room temperature. A solution of 50/50 (v/v) ethanol/water (5 mL) was then added to the mixture and stirred well. Upon formation of the colored complex, known as Ruhemann's purple, the absorbance at 570 nm was measured. The amount of functional aldehyde groups was



Scheme 1: Chemical structure of commercial carrier Eupergit®C.



Scheme 2: Functionalization steps of Eupergit®C with ethylenediamine and glutaraldehyde.

calculated using the following equation:

$$\rho\ (\%) = (A/B) \times 100 \quad (1)$$

ρ is the percentage of-NH_2 groups on the support surface after the glutaraldehyde-modified Eupergit®C-NH_2; **A** is the absorbance recorded for the ninhydrin reagent/ Eupergit®C-NH_2-CHO mixture;

B is the absorbance recorded for the mixture ninhydrin reagent/Eupergit®C-NH_2.

2.3 Lipase immobilization

Glutaraldehyde-modified Eupergit®C (2 g) was incubated with lipase solution (8 mL; 1 mg/mL) prepared in sodium phosphate buffer (100 mM, pH = 6.5) (Scheme 3). The reaction was allowed to continue in a water-bath at 10 °C for 2 h with slow shaking. After the enzyme immobilization step (Eupergit®C-NH_2-CHO-Lip), the unbound enzyme was removed by washing the support three times with the same buffer and stored at 5 °C.

The enzyme immobilization efficiency (EF) was calculated using the following equation:

$$EF(\%) = (Ai/Af) \times 100 \quad (2)$$

where *Ai* is the specific activity of immobilized enzyme, equal to the difference between the specific activity of free enzyme solution (*Af*) and the specific activity of the unbound enzyme in the washing buffer.

The enzyme loading amount (Q_e, mg/g dry carrier) was calculated according to the following equation:

Q_e (mg/g) = added Lipase (mg) – unbound Lipase (mg) / carrier weight (g).

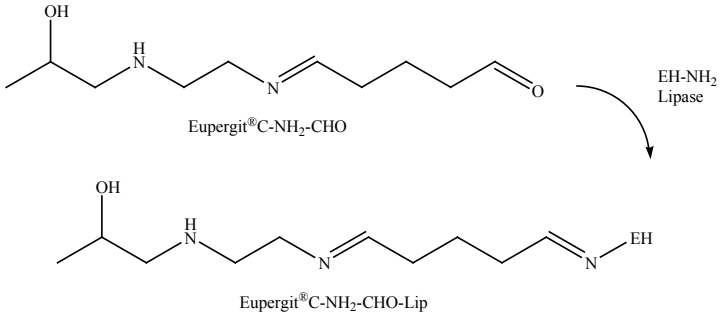
2.4 Assay of lipase activity

Lipase specific activity was performed as described in the literature to verify enzyme immobilization efficiency [26]. A p-nitrophenyl butyrate solution (0.62 M) in acetonitrile was prepared and then mixed with a solution of acetonitrile and buffer (50 mM, pH 4-7.5) to obtain the appropriate substrate solution [27]. Then, the substrate solution was incubated with lipase. After incubation at 60 °C for 15 min., the lipase activity was measured by monitoring the absorbance at 405 nm as experimental evidence of p-nitrophenol formation [28]. The lipase-mediated hydrolysis reaction of p-nitrophenyl butyrate is given in Scheme 4. One unit of activity was defined as the amount of enzyme to form 1 µmol of p-nitrophenol per minute under the assay conditions.

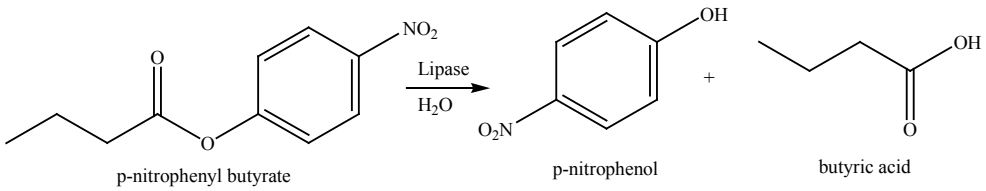
2.5 Lipase characterization

The effect of pH on the activity of the free and immobilized lipase was studied by mixing the enzyme with 0.1 M sodium phosphate buffer with pH ranging from 4 – 7.5, sodium hydrogen phosphate buffer for the pH range 4 – 5.5, and sodium dihydrogen phosphate buffer for the pH range 6 – 7.5; the temperature was maintained at 35 °C. The effect of temperature on lipase activity (for the free and immobilized form) was studied in the 0.1 M sodium phosphate buffer (at the optimal pH) at temperatures from 25 to 60 °C.

The operating stability of the immobilized lipase was assessed as follows: several consecutive operating cycles were performed by the esterification reaction at the



Scheme 3: Lipase immobilization onto glutaraldehyde-modified Eupergit®C.



Scheme 4: Hydrolysis reaction of p-nitrophenyl butyrate catalyzed by lipase from *Candida rugosa*.

optimal temperature and pH. At the end of each reaction cycle, the immobilized lipase was washed once with hexane, dried for 15 minutes at 40 °C, and the procedure was repeated in a new batch reaction.

2.6 Esterification reaction

Ethyl butyrate synthesis was initially carried out in the absence of organic solvent at the temperature corresponding to the optimal activity of the free and immobilized lipase. The reaction mixture was incubated with 50 mg of Eupergit®C-NH_2-CHO-Lip on a rotary shaker at 150 rpm. The effect of the acid-alcohol molar ratio was studied for different experimental conditions: this entailed molar ratios of 1:1, 1:2, 1:5, 5:1, and 2:1 and reaction times of 4, 12, 24 and 48 h. The reaction total volume was 1 mL.

The effect of the enzyme amount loaded onto 50, 100 and 200 mg of Eupergit®C-NH_2-CHO-Lip was monitored after 48, 72, 96 and 120 h of reaction. This effect was also evaluated for the same amount of free enzyme in order to compare its catalytic activity with the immobilized form. Finally, the esterification reaction was tested in hexane. Hexane is commonly used as a solvent for enzymatic reactions in organic media because it is cheap, non-toxic to the enzyme, and used in the food industry.

The conversion percentage (%) of ethyl butyrate was measured by determining the unreacted butyric acid in the reaction mixture by titration with 2 M NaOH, using phenolphthalein as an indicator. All the experiments were repeated in triplicate using the following equation:

$$\text{Conversion } (\%) = (R–C)/R \quad (3)$$

R is the volume of NaOH in the reference reaction mixture (ethanol and butyric acid) and **C** is the volume of NaOH used in the reaction mixture after the incubation with lipase.

2.7 Infrared spectroscopy

Infrared spectroscopy was used for the characterization of the modified support Eupergit®C-NH_2-CHO-Lip and ethyl butyrate. The IR spectra were recorded with the Agilent Technologies Cary 630 FT-IR spectrometer (ATR technique) at 2 cm^{-1} resolution. Microlab PC FT-IR and Varian Resolution Pro 410.101 software were used for data acquisition and processing, respectively.

3 Results

3.1 Carrier characterization

3.1.1 Infrared spectroscopy

The functionalization steps of Eupergit®C with ethylenediamine and glutaraldehyde, and immobilization of the lipase, were analyzed by infrared spectroscopy. The IR spectra of unmodified Eupergit®C and Eupergit®C-NH_2-CHO-Lip are shown in Fig.1.

The IR spectrum of unmodified Eupergit®C (Fig. 1a) showed a broad band at ~ 3300 cm^{-1} due to NH bond stretching; the methylene group vibration was observed at 2933 cm^{-1} and 1484 cm^{-1} (bending). Amide groups were identified by the bands at 1633 cm^{-1} (C=O stretching) and 1563 cm^{-1} (NH bending). The stretching of the C-O-C bond gave rise to bands at 1194 cm^{-1} and 1082 cm^{-1}. The characteristic bands related to the epoxy groups occurred in the spectrum range 980 – 800 cm^{-1}.

In Fig. 1b, the IR spectrum of the modified support Eupergit®C-NH_2-CHO-Lip is reported. A band at 1068 cm^{-1}, due to the stretching of the C=N bond formed between the carbonyl group of glutaraldehyde and the lipase amino groups, was evident.

The IR spectra of amino-functionalized Eupergit®C before (Eupergit®C-NH_2) and after glutaraldeyde activation (Eupergit®C-NH_2-CHO) are reported as Supplemental data (Fig. S1 and S2).

3.1.2 Ninhydrin test and enzyme immobilization efficiency

The ninhydrin test is a useful method to determine the effectiveness of modification of Eupergit®C-NH_2 with glutaraldehyde [25]. The percentage of ethylenediamine linked covalently to glutaraldehyde is directly related to the amount of enzyme to be immobilized onto the support. The UV-visible spectrum, in relation to the colored complex known as “purple Ruheumann,” is shown in Fig 2. The amount of ethylenediamine linked to glutaraldehyde was estimated by recording a less intense absorption band at 570 nm with respect to that recorded for the mixture with the support Eupergit®C-NH_2-CHO. In this case, a reduction of 81.5% of the absorption band was observed after the glutaraldehyde addition. This value was in agreement with enzyme immobilization efficiency, calculated to be 85%. The protein loading amount was ~ 3.4 mg/g carrier (3468 U/g carrier).

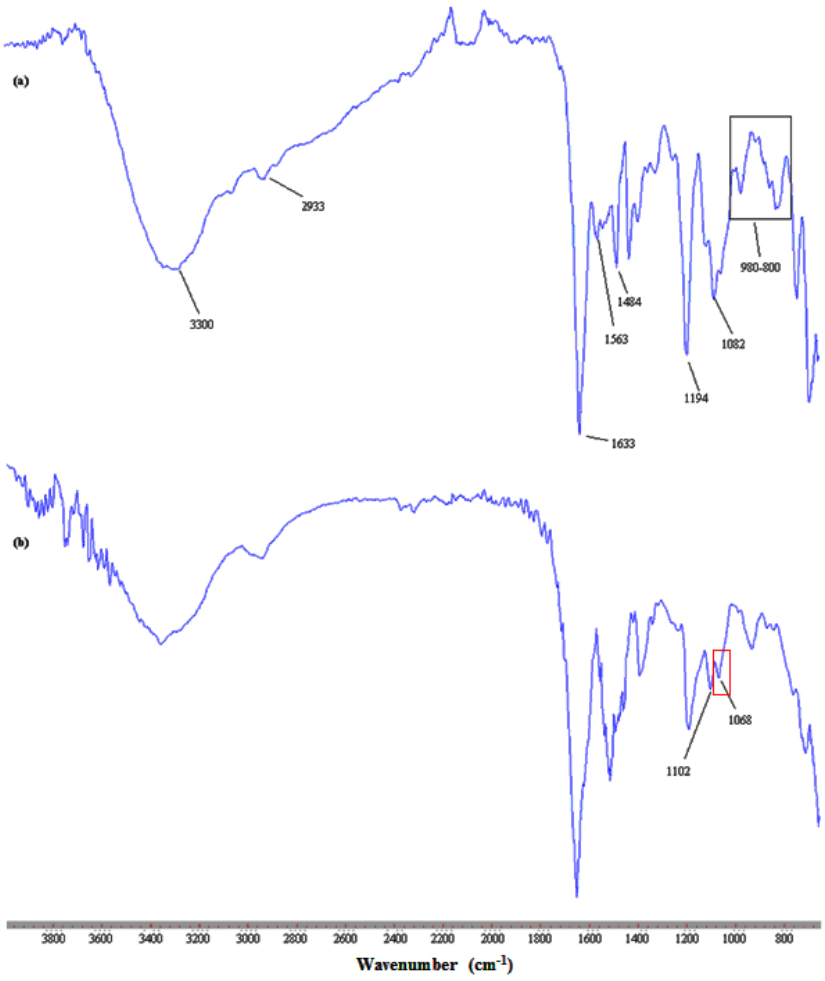


Figure 1: IR spectrum of unmodified Eupergit®C (a) after lipase immobilization (b) the black and red boxes highlight the characteristic bands of the Eupergit®C epoxide group (~980-800 cm^{-1}) and C=N bond formation (1068 cm^{-1}) following immobilization.

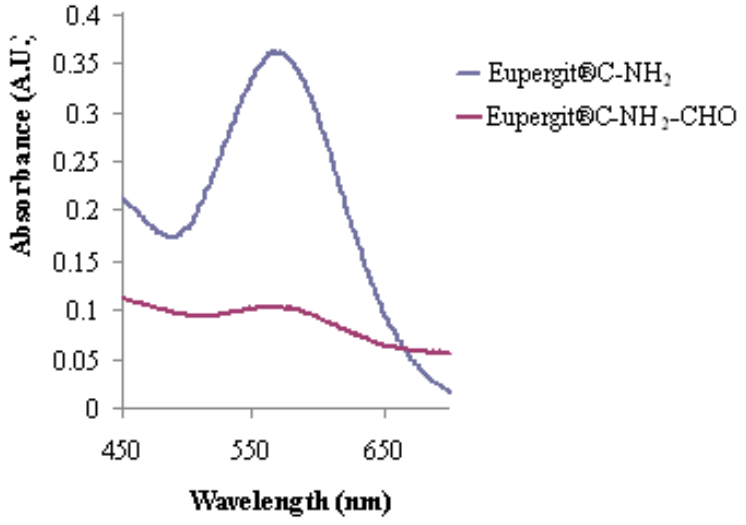


Figure 2: UV-visible spectrum of modified Eupergit®C with ethylenediamine (Eupergit®C-NH_2) and glutaraldehyde (Eupergit®C-NH_2-CHO).

3.2 Characterization of free and immobilized lipase

The catalytic properties of the free and immobilized lipase were studied under various pH conditions and temperatures. The optimum pH for the enzymatic activity of free lipase was in the range 6.0 – 6.5 and the maximum activity for the immobilized lipase was found to occur at a pH of 6.5 (Fig. 3). As a result of the immobilization process, the catalytic activity of the immobilized enzyme was maintained around 90%, while, for the free lipase, a significant activity decrease to 40% was detected. The temperature-activity profile of the free and the immobilized lipase was investigated to determine the matrix-protecting effect on the immobilized enzyme with respect to thermal denaturation. Maximum activities were observed at 40 °C for the free and immobilized enzyme, as reported in Fig. 4. The temperature profile indicated a higher activity for the immobilized enzyme (55 – 60 °C) compared to that of the free form.

3.3 Synthesis of ethyl butyrate in solvent free-system

3.3.1 Effect of acid-alcohol molar ratio

The effects of different acid-alcohol molar ratios were investigated using free and immobilized lipase in a solvent-free system. One strategy to shift the equilibrium of the reaction in the forward direction is to increase the alcohol (nucleophile) concentration. Therefore, it is necessary to optimize the excess nucleophile concentration. In order to study the effect of acid:alcohol molar ratio on the ethyl butyrate synthesis, experiments were performed using the equivalent amount of enzyme (in the free and supported form) and monitored for 4, 12, 24 and 48 h. An optimal temperature of 40 °C was chosen for the ester synthesis with the immobilized lipase. Figure 5 illustrates the change in conversion as a function of the reaction time at different acid:alcohol molar ratios (1:1, 1:2, 2:1, 1:5 and 5:1) for free and immobilized lipase.

According to the low biocatalyst amount, the maximum ester yields were ~ 6% and ~ 10% for free and immobilized lipase, respectively, at an acid:alcohol molar ratio of 1:2. Furthermore, the ester yield increased with increasing acid:alcohol molar ratio up to a threshold value.

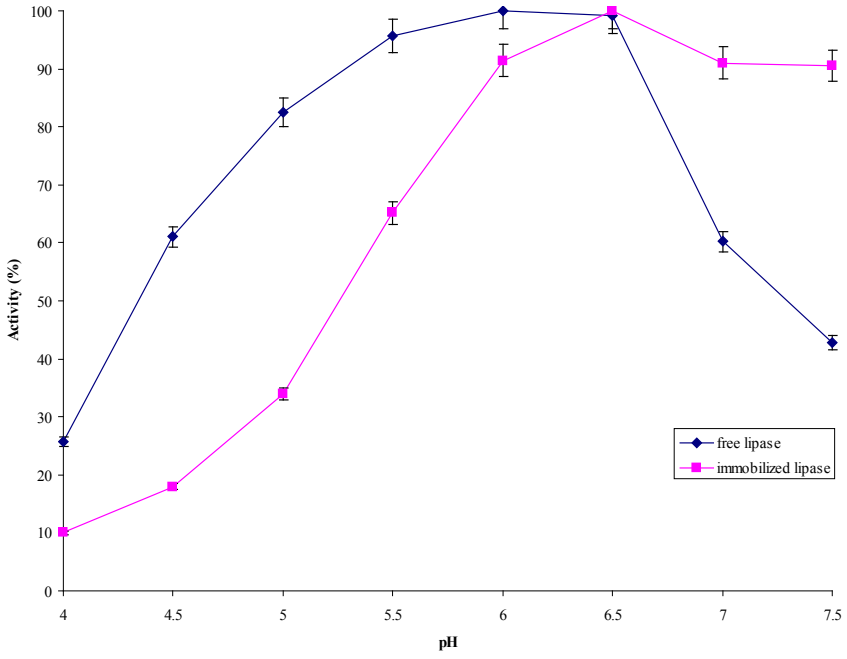


Figure 3: Effect of pH on activity of free (◆) and immobilized lipase (■). Experiments were performed in triplicate. The error bars indicate the standard deviation (3%).

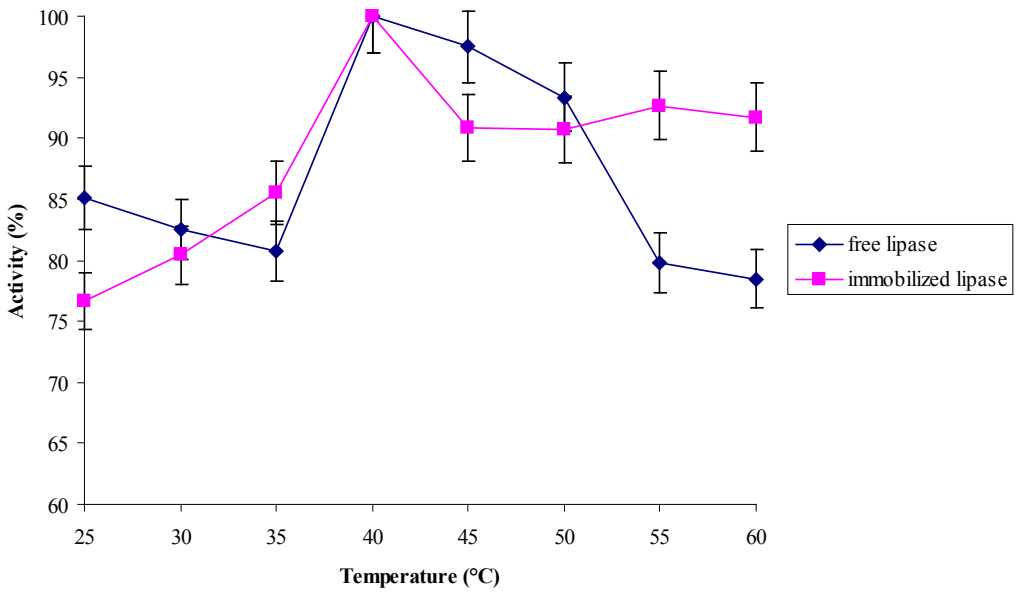


Figure 4: Effect of temperature on free (◆) and immobilized lipase (■). Experiments were performed in triplicate. The error bars indicate the standard deviation (3%).

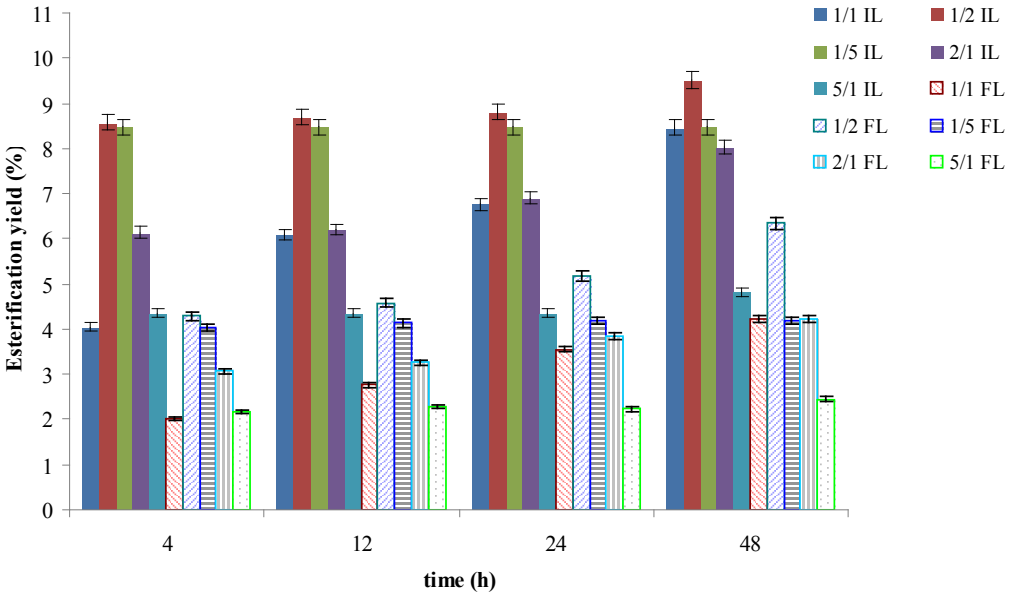


Figure 5: Effect of acid/alcohol molar ratio on the ester conversion percentage using free (FL, empty bars) and immobilized lipase (IL, filled bars) in solvent-free system. The error bars indicate the standard deviation (2%).

3.3.2 Effect of the enzyme concentration

The effect of enzyme concentration was studied for the 1:2 ratio using free lipase (0.2 mg, 0.4 mg and 0.8 mg) and the same amount of immobilized lipase (corresponding to around 50 mg, 100 mg and 200 mg of the supported enzyme) in a solvent-free system at 40 °C. Fig. 6 shows the effect of enzyme concentration on ethyl butyrate synthesis after 48, 72, 96 and 120 h. The ester yield increased with the amount of enzyme in the reaction medium for both the free and immobilized lipase. Using 200 mg of the enzyme-support system (equivalent to 0.8 mg of free lipase), the ester conversion percentage was ~ 23%. These results indicate that use of the immobilized enzyme leads to higher ester yields than the same amount of free lipase.

3.4 Synthesis of ethyl butyrate in organic solvent

The use of a lipase in organic media, rather than aqueous, shifts the reaction in favor of product formation. Therefore, starting from the previously optimized experimental conditions (200 mg of the enzyme-support system and 1:2 acid:alcohol molar ratio), this effect was investigated for the esterification reaction using hexane as a solvent. In Fig. 7 the reaction yield is reported for 4, 8, 12, 24, 48, 96 and 120 h; a maximum yield of 65% was obtained after 120 h. The reaction profile revealed a slow esterification reaction paired with a good product yield compared to the synthesis in solvent-free system.

The effect of repeated use on the activity of the immobilized lipase can be observed in Fig. 8, which shows a decrease in the activity of the immobilized enzyme after several reuses. The residual activity of lipase immobilized onto modified Eupergit®C was ~ 60% after 8 cycles of batch reactions.

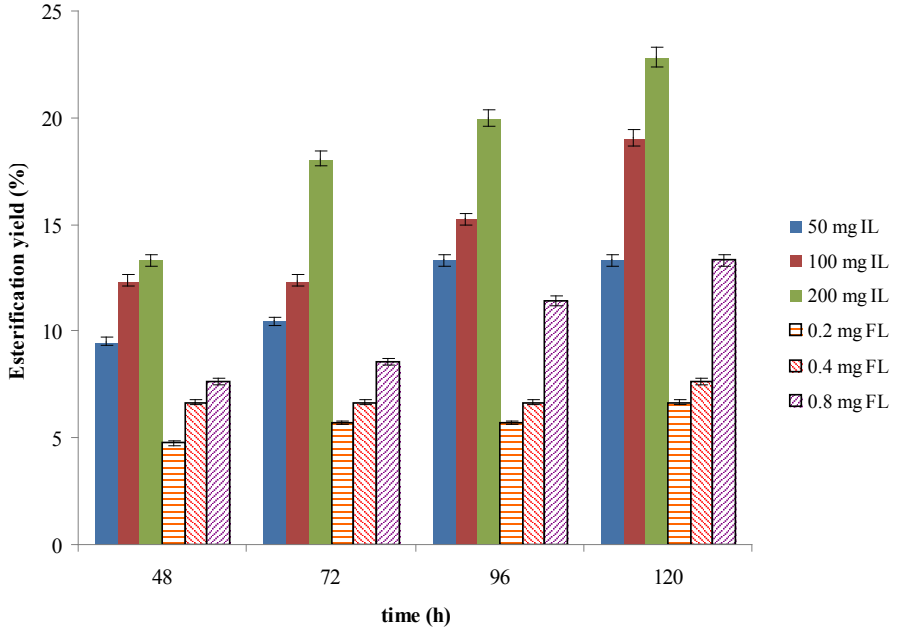


Figure 6: Effect of enzyme amount on the ester conversion percentage in a solvent-free system: free lipase (FL, empty bars) and immobilized lipase (IL, filled bars). The error bars indicate the standard deviation (2%).

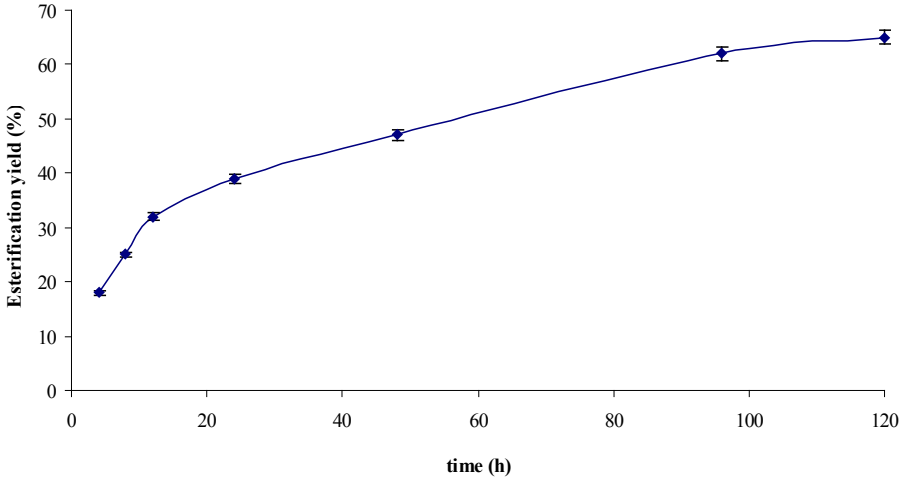


Figure 7: Effect of organic solvent (hexane) on the ester conversion percentage using immobilized lipase. The error bars indicate the standard deviation (2%).

3.5 Product characterization

The product obtained from the esterification reaction was characterized by IR spectroscopy (Fig. 9). A weak band due to the O-H groups of ethanol and butyric acid was observed at ~ 3000 cm^{-1}; in this region of the spectrum, a series of bands appearing in the range of 2980 to 2850 cm^{-1} were attributed to C-H stretching. The band occurring at 1715 cm^{-1} was associated with the characteristic C=O stretching vibration of esters, while bands at 1190 cm^{-1} were assigned to the C-O-C bending modes of the ester bond. Finally, those appearing at 1095 and 1050 cm^{-1} were assigned to the C-O stretch, confirming the formation of ethyl butyrate.

4 Discussion

Eupergit®C is particularly attractive for enzyme immobilization because it can be used in extreme reaction conditions due to its excellent mechanical properties and chemical stability [20]. As reported in the literature, direct lipase immobilization on Eupergit®C is possible, but a significantly lower catalytic efficiency is detected with respect to the use of a crosslinker such as glutaraldehyde [29]. Immobilization methods with molecules such as glutaraldehyde indeed provide the appropriate distance between the support and the enzyme, increasing the catalytic activity [29]. For this reason, ethylenediamine (as a spacer) and

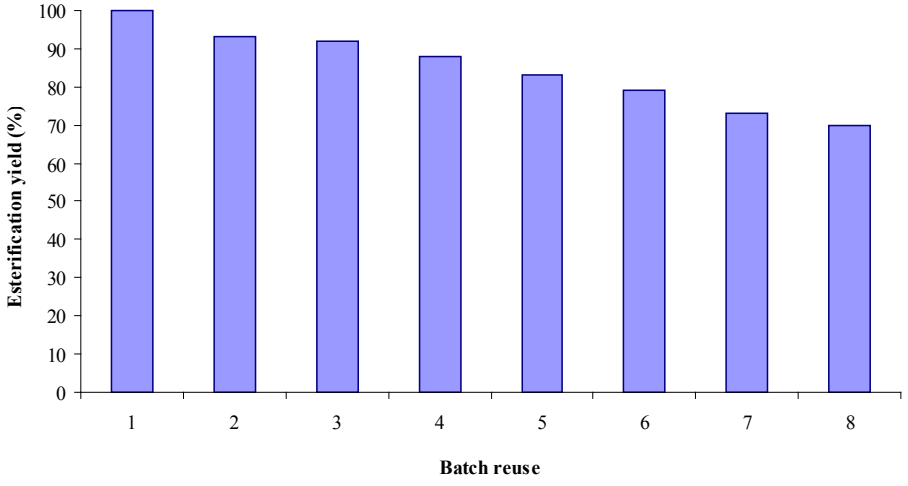


Figure 8: Operational stability of immobilized lipase in organic solvent over repeated batch reactions. The error bars indicate the standard deviation is equal to 2%.

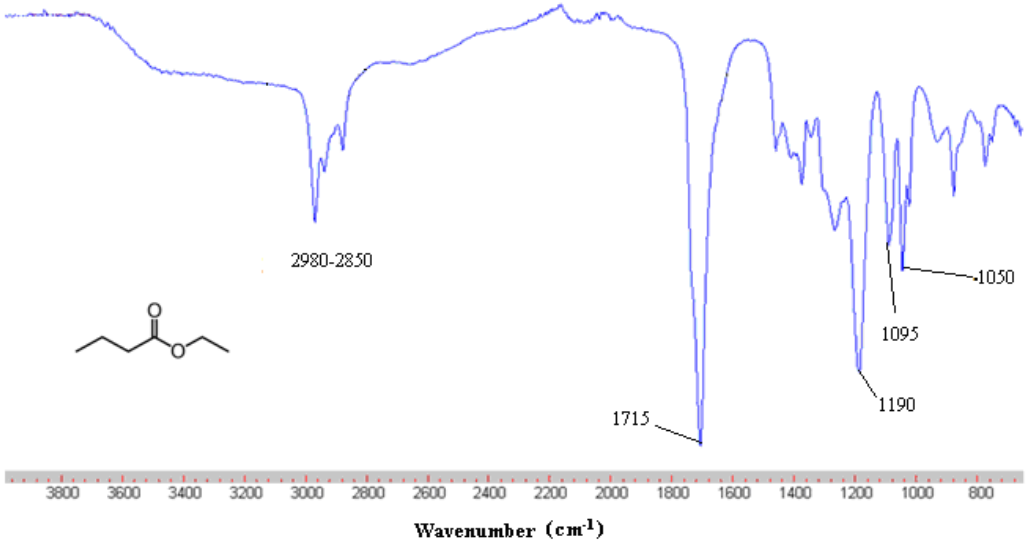


Figure 9: IR spectrum of ethyl butyrate obtained by immobilized lipase onto modified Eupergit®C.

glutaraldehyde (as a crosslinker) have been used in this study. Furthermore, as glutaraldehyde is a bifunctional molecule, two possible mechanisms for its reaction with ethylenediamine are possible: (1) each molecule of glutaraldehyde could be bound to two molecules of ethylenediamine, thus becoming inactive for the enzyme immobilization, and (2) the formation of ethylenediamine-glutaraldehyde bonds with the subsequent formation of glutaraldehyde-enzyme bonds. In our study, the enzyme immobilization efficiency was calculated to be 85%, suggesting the predominant formation of ethylenediamine-glutaraldehyde bonds; this conclusion was also confirmed by a ninhydrin test.

The results obtained for pH and thermal stability of the immobilized enzyme show an improved activity, which is in agreement with the reports for immobilization of other enzymes [30-32].

A key parameter in the synthesis of flavor esters by immobilized enzymes is their reusability; this allows the biocatalytic system to become cost-competitive with traditional chemical processes. Usually, enzyme activity decreases after several reuses because of enzyme desorption, enzyme deactivation, or inhibition caused by substrates or products [33]. In this study, a good residual activity (~ 60%) has been verified for the immobilized lipase onto modified Eupergit®C after 8 batch reactions. These results are also consistent with a study on pineapple flavour synthesis by immobilized lipase from *Rhizomucor miehei*, in which washing of the lipase-support system with the solvent, after each batch reaction, demonstrated a positive effect on the stability of the enzyme [33]. Furthermore, operational stability is also a key parameter for other industrial enzymes such as laccase from *Trametes versicolor* immobilized onto Amberlite IR-120H and nylon carriers [34,35].

Several parameters can affect the product conversion in the enzymatic esterification. The reactions are reversible and the presence of water, as a byproduct, leads to a decrease in ester yield, as it displaces the equilibrium towards the reactants. Therefore, ester synthesis can be induced using the following methods: (1) removing the water by the use of desiccants or other suitable technologies [36], or (2) conducting the reaction by appropriate molar ratios between carboxylic acid and alcohol. As reported in several studies, indeed, acids may cause acidification of the microaqueous interface leading to enzyme inactivation [24,37].

The use of ethanol can lead to lower esterification yields for its tendency to strips off from the reaction media [38]. Conversely, use of long chain alcohols like butanol and pentanol could increase ester conversion yields for most combinations with short chain acids [39]. In any case, the low product yield obtained in the proposed solvent-free system has also been observed for lauryl palmitate synthesis (long chain ester) using commercial Novozym 435 [40]. On the other hand, as demonstrated in this study, the use of a lipase in organic rather than aqueous media shifts the reaction in favor of product formation and increases the solubility of polar substrates such as ethanol [41]. Moreover, lipase catalysis in organic media confers other advantages: easy enzyme and product recovery, the occurrence of new reactions not possible in water because of kinetic or thermodynamic restrictions (i.e., the water effect in transesterification reactions), and increased enzyme stability [42].

In general, the use of a supported enzyme improves the catalytic activity of the enzyme itself. Similar results have been obtained for *P. aeruginosa* 42A2 lipase immobilized onto a polypropylene matrix Accurel MP1000: a lower yield of Fatty Acid Methyl Esters (FAMEs) was obtained with free enzyme in comparison to that obtained by immobilized lipase [43]. Therefore, the immobilized lipase biocatalyst demonstrated a good performance for the synthesis of short-chain esters. The choice of a suitable support is a key aspect for the application of enzymes in industry, and new nanostructured materials may indeed show interesting results for the development of novel enzymatic supports.

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